



**University of
Zurich^{UZH}**

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2014

Determination of uromodulin in human urine: influence of storage and processing

Youhanna, Sonia ; Weber, Julien ; Beaujean, Viviane ; Glaudemans, Bob ; Sobek, Jens ; Devuyst, Olivier

Abstract: **BACKGROUND:** Uromodulin (Tamm-Horsfall protein) is the most abundant protein excreted in the urine under physiological conditions. It is exclusively produced in the kidney and secreted into the urine via proteolytic cleavage. The involvement of UMOD, the gene that encodes uromodulin, in rare autosomal dominant diseases, and its robust genome-wide association with the risk of chronic kidney disease suggest that the level of uromodulin in urine could represent a critical biomarker for kidney function. The structure of uromodulin is complex, with multiple disulfide bonds and typical domains of extracellular proteins. **METHODS:** Thus far, the conditions influencing stability and measurement of uromodulin in human urine have not been systematically investigated, giving inconsistent results. In this study, we used a robust, in-house ELISA to characterize the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in the urine. **RESULTS:** The levels of uromodulin in human urine were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage. **CONCLUSIONS:** These results validate a simple, low-cost ELISA and document the optimal conditions of processing and storage for measuring uromodulin in human urine.

DOI: <https://doi.org/10.1093/ndt/gft345>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-83925>

Journal Article

Accepted Version

Originally published at:

Youhanna, Sonia; Weber, Julien; Beaujean, Viviane; Glaudemans, Bob; Sobek, Jens; Devuyst, Olivier (2014). Determination of uromodulin in human urine: influence of storage and processing. *Nephrology, Dialysis, Transplantation*, 29(1):136-145.

DOI: <https://doi.org/10.1093/ndt/gft345>

NDT-00360-2013.R1

Determination of Uromodulin in Human Urine: Influence of Storage and Processing

Sonia Youhanna^{1*}, Julien Weber^{1*}, Viviane Beaujean³, Robertus Glaudemans¹, Jens Sobek²,
and Olivier Devuyst^{1,3}

¹Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich,
Switzerland; ²Functional Genomics Center Zurich, Switzerland; ³Division of Nephrology,
Université catholique de Louvain Medical School, Brussels, Belgium.

* SY and JW contributed equally to this study

RunningTitle: Uromodulin in human urine

Correspondence: to OD, Institute of Physiology, University of Zurich, Switzerland; E-mail:
olivier.devuyst@uzh.ch

ABSTRACT

Uromodulin (Tamm-Horsfall protein) is the most abundant protein excreted in the urine under physiological conditions. It is exclusively produced in the kidney and secreted into the urine via proteolytic cleavage. The involvement of UMOD, the gene that encodes uromodulin, in rare autosomal dominant diseases, and its robust genome-wide association with the risk of chronic kidney disease suggest that the level of uromodulin in urine could represent a critical biomarker for kidney function. The structure of uromodulin is complex, with multiple disulfide bonds and typical domains of extracellular proteins. Thus far, the conditions influencing stability and measurement of uromodulin in human urine have not been systematically investigated, giving inconsistent results. In this study, we used a robust, in-house ELISA to characterize the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in the urine. The levels of uromodulin in human urine were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage. These results validate a simple, low-cost ELISA and document the optimal conditions of processing and storage for measuring uromodulin in human urine.

INTRODUCTION

Urinary biomarkers constitute an essential tool for the diagnosis, classification, and prognosis of kidney diseases (1). Recent evidence pointed at uromodulin (originally named Tamm-Horsfall glycoprotein) as a potential urinary biomarker relevant for renal function, chronic kidney disease (CKD) and hypertension (2,3). Uromodulin is a 105 kD glycoprotein with seven N-glycosylation sites and a high-mannose chain. The protein contains 616 amino acids including 48 cysteine residues that are all engaged in the formation of disulfide bonds. Uromodulin contains three epidermal growth factor (EGF)-like domains and a zona pellucida (ZP) domain, found in many extracellular proteins, as well as a glycosylphosphatidylinositol (GPI)-anchoring site (3). Uromodulin is a kidney-specific protein, that is exclusively synthesized in the epithelial cells lining the thick ascending limb (TAL) of Henle's loop (4). After proper trafficking and maturation in TAL-lining cells, uromodulin reaches the apical plasma membrane, to be cleaved and assembled in the urine as polymers forming a gel-like structure (5).

Uromodulin is produced at very high rate in the TAL, and is by far the most abundant protein in normal urine (excretion: 50-100 mg/day) (6). Functions attributed to uromodulin include protection against urinary tract infections; prevention of renal calculi formation by reducing aggregation of calcium crystals; and influencing transport processes by regulating the activity of NKCC2 and/or ROMK (7,8). Interest for uromodulin was re-ignited when it was discovered that mutations in the UMOD gene that codes for uromodulin are responsible for a series of monogenic disorders (familial juvenile hyperuricemia nephropathy, medullary cystic kidney disease type 2 or glomerulocystic kidney disease) all known as uromodulin-associated kidney disease (UAKD) (3). These disorders are characterized by severe tubulointerstitial damage, defective urinary concentration, hyperuricemia and gout, and progressive renal failure

(9). The mutations often affect cysteine residues, resulting in conformational changes and intracellular aggregates of uromodulin. In turn, there is a strong decrease in the secretion of the protein by the TAL cells and a strong decrease in the urinary excretion of uromodulin (10-12). Lately, a number of genome wide association studies (GWAS) revealed that variants in the UMOD gene were strongly associated with markers of renal function and risk of developing hypertension and CKD in the general population (13-15). The association of uromodulin with both monogenic diseases and complex disorders such as CKD and hypertension provides a strong rationale for evaluating its urinary concentration as biomarker for renal function and CKD.

The determination of uromodulin in the urine is hampered primarily by its capacity to aggregate and the potential instability of such a complex protein. Documentation of sampling, processing and storage conditions is thus crucial for accurate uromodulin quantification. Despite the early characterization of antibodies specific for human uromodulin (16), the few reports on uromodulin dosage yielded conflicting results in terms of stability, storage conditions and processing of human urine (17-20). Important points such as the potential influence of urine centrifugation or vortexing, acidification or alkalization, treatment with protease inhibitors, or normalization for urinary creatinine remain unsolved. Furthermore, earlier immunoassays were often based on poorly documented anti-uromodulin antibodies. Considering the increasing interest for a robust determination of uromodulin in the urine, the need for a high-throughput assay, and the limited and contradictory information available, we developed and characterized a robust ELISA for uromodulin and used this assay to investigate the stability of uromodulin under different treatment and storage conditions of human urine.

MATERIAL AND METHODS

Urine sample collection, storage and handling

Analyses were performed on second morning urine samples collected (mid-stream) in a sterile container from healthy volunteers aged 18-50 years, and processed within 2 h. This protocol was approved by the Ethical Committee of the Université catholique de Louvain.

The influence of human urine sample processing on the determination of uromodulin ([Fig.1](#)) was tested after vortexing the sample for 10 sec (Vortex-Genie 2, FAUST, Schaffhausen, Switzerland); centrifugation for 10 min at 3,600 rpm (Eppendorf Centrifuge 5430, Hamburg, Germany) at room temperature (i.e. standard protocol for urine processing and removing cells and debris; ref. 21); treatment with protease inhibitors (Leupeptin 1 μ mol/L; Sigma-Aldrich, St. Gallen, Switzerland; sodium azide 10 mmol/L); pH adjustment performed by drop titration with 1N HCl (to pH 2.0) or with 1N NaOH (to pH 8.0) using a Hanna HI 2211 pH meter; dilution using ultrapure deionized water (Destamat Bi 18E, QCS, Maintal, Germany) vs. TEA buffer (0.5% Triton X-100, 20 mM EDTA, pH 7.5). The effect of storage conditions was tested by comparing baseline levels with 1-week and 5-month storage at room temperature, +4°C and -20°C; 4-month and 8-month storage at -80 °C; five cycles of freezing-thawing (sample kept at -80 °C for 48 h followed by thawing on ice). Different sample sets were used to evaluate the influence of the various processing conditions as described.

Uromodulin ELISA

The in-house ELISA for uromodulin is a colorimetric based sandwich immunoassay using a sheep anti-human uromodulin antibody (Meridian Life Science, Memphis, USA; K90071C) as

the capture antibody. This antibody gives a single arc when tested by immuno-electrophoresis against fresh urine. The primary antibody was a monoclonal anti-human uromodulin antibody (Cedarlane Laboratories, Burlington, USA; CL 1032A) raised in mouse and validated in solid phase radioimmunoassay. The secondary antibody was a goat anti-mouse IgG (H+L) horseradish peroxidase conjugated (Bio-Rad, Cressier, Switzerland; 172.1011). The substrate was O-Phenylenediamine dihydrochloride (OPD) (10mg/tablet) (Sigma-Aldrich). The OPD substrate solution was freshly prepared by dissolving a tablet in 25 mL of phosphate-citrate buffer [0.1 M citric acid monohydrate, 0.2 M Na₂HPO₄], pH 5.5. A volume of 5 µL 30% H₂O₂ was added to 25 mL of substrate solution. Human uromodulin (Millipore, Billerica, USA) was used to establish the standard curve, with freshly prepared serial dilutions from the standard stock solution (100 µg/mL). Both the standard curve and a standard sample (uromodulin concentration 25 µg/mL) were systematically used for quality control (QC).

The determination of urinary uromodulin by ELISA was carried out as follows: a 96-well microtiter plate (NUNC MaxiSorp™, eBioscience, Vienna, Austria) was coated with 100 µL of 5 µg/mL capture antibody in coating buffer [500 mM H₃BO₃, 500 mM KCl, 345 mM NaOH, pH 9.0]. The plate covered with adhesive seal was incubated at 4°C overnight then washed three times with freshly prepared washing buffer (0.1 % Tween 20 in 10 mM phosphate buffer saline (PBS) pH 7.2 (PBS-Tween 0.1%)) using ImmunoWash 1575 Microplate Washer (Bio-Rad). Unoccupied sites on the plate were blocked with 100 µL blocking buffer (0.5 % BSA in 10 mM PBS, pH 7.2) and incubated at 37°C for 1 h with a slow rotation (100 rpm). The plate was then washed three times with washing buffer and placed upside-down on absorbent paper to remove residual buffer. 100 µL of PBS-Tween 0.1 % was dispensed in all wells. Urine samples were stabilized at room temperature then diluted 1:50 in ultrapure deionized water, as preliminary

testing revealed no significant difference vs. dilution with TEA buffer (data not shown). A volume of 100 μ L per well was distributed into the coated wells after vortexing. Standards and QC sample were run in duplicate whereas each urine sample was tested in 3 different dilutions. Deionized water was used as blank. After 1 h incubation at 37°C, the plate was washed three times and placed on absorbent paper. 100 μ L of primary antibody diluted in PBS-Tween 0.1% (1 μ g/mL) was dispensed in each well; the plate was incubated at 37°C for 1 h (rotation, 100 rpm) then washed three times. The secondary antibody diluted 1:2,000 in PBS-Tween 0.1% was added to the wells for 45 min at 37°C and the plate washed three times. Color was developed by adding 100 μ L of OPD substrate solution. The plate was incubated at room temperature in the dark for 1 min, and the reaction stopped by adding 50 μ L of 2M H₂SO₄ to each well. Optical density (Infinite M200Pro, Tecan; Grödig, Austria) was read at 492nm and urinary uromodulin concentration was determined by referring to the standard curve. Uromodulin levels obtained using the in-house ELISA were compared to the commercial ELISA from MD Bioproduct (St. Paul, USA; M036020), following the protocol given by the manufacturer. This test has been used in several studies (13,22). Urinary creatinine levels (normalization) were measured using the Synchron® System Creatinine Assay (Unicell DxC Synchron®, Beckman Coulter, Brea, USA), following the manufacturer instructions.

Immunoblotting

Kidneys from Umod mice (23) were grounded in liquid nitrogen and homogenized as described previously (12). The homogenate was centrifuged at 1000 \times g for 15 min at 4°C and the resulting supernatant at 100,000 \times g for 120 min at 4°C. The pellet was suspended in homogenization buffer before determination of protein concentration (Pierce BCA protein assay kit; Thermo

Fischer Scientific; Rockford, USA). SDS-PAGE for mouse and human samples was performed under reducing conditions. Samples (20 µg of mouse and human kidney extract; 2µL of urine) were loaded after being mixed with Laemmli sample buffer and heated for 5 min at 95°C (kidney samples). Proteins were separated on 10 % SDS gel and transferred to nitrocellulose membrane for Western blotting. Membranes were blocked with 5 % milk blot for 30 min at room temperature then incubated overnight at 4°C with either sheep or mouse anti-uromodulin antibodies (1:400 in 0.5% BSA blocking buffer). Secondary antibodies were goat anti-mouse HRP conjugated (1:10,000) or polyclonal rabbit anti-sheep HRP conjugated (1:1,000), for 1 h at room temperature. Antigen-antibody reaction was detected by using ECL (Immun-Star HRP, Bio-Rad) and light-sensitive film (GE Healthcare, Glattbrugg, Switzerland). The molecular weight was estimated by running the Precision Plus Protein™ All Blue standard (Bio-Rad).

Deglycosylation and desialylation of uromodulin

Deglycosylation of uromodulin from human urine was carried out using PNGase F (PNGase F P0704S, New England Biolabs, Ipswich, USA) following the manufacturer's protocol, whereas desialylation was performed according the protocol described by Parsons et al. (24). Briefly, uromodulin was precipitated from pooled human urine (1.5L) following the protocol of Tamm and Horsfall (2), dialyzed overnight at 4°C and then lyophilized (Virtis, Kloten Switzerland). Dry uromodulin was solubilized in 2.5M acetic acid (10 mg/ml), heated for 3 h at 82 °C, then washed 3 times with 15 mL PBS (pH 7.2) on Centricon (MWCO 30000) cartridge (Millipore). 1.5 µL of deglycosylated urine and 0.1 µL of desialylated uromodulin (vs. 0.5 µL of untreated urine) were loaded on 10 % acrylamide gel and analyzed as described above.

Immunohistochemistry

Colocalization of uromodulin with NKCC2 was carried out in cryosections of human and mouse kidney samples as previously described (10,12). Briefly, 5 µm-thick cryosections were blocked with 1% BSA/0.02% sodium azide-PBS for 30 min at room temperature, incubated for 2 h at room temperature with the sheep (1:400) or mouse (1:200) antibodies against human uromodulin, followed by washing and incubation with AlexaFluor633-conjugated donkey anti-sheep or goat anti-mouse (1:200) for 90 min at room temperature. Uromodulin-stained sections were then incubated with a polyclonal rabbit anti-NKCC2 antibody (Millipore; AB3562P; 1:100) for 3 h at room temperature, followed by washing and incubation with Alexafluor488-conjugated goat anti-rabbit antibodies (1:200). Sections were viewed on a Leica SP5 confocal microscope.

Surface plasmon resonance: Biacore

The interaction between uromodulin and the capture antibody was analyzed by surface plasmon resonance, using a Biacore T100 system (GE Healthcare, Uppsala, Sweden). Chemicals were from Sigma unless otherwise noticed. Binding experiments were performed in PBS buffer pH 7.4 containing 0.2% of Tween 20 at a flow rate of 30 µL/min at 25°C. Ultrapure and filtered water (“MilliQ”, Millipore, Billerica, USA) was used for preparing all solutions. The carboxymethyl dextran chip (CMD500L, XanTec bioanalytics, Düsseldorf, Germany) surface (1.2 mm² area) was cleaned before use by injecting 7 times a 50 mM NaOH solution containing 1 M NaCl for 30 sec at a flow rate of 5 µL/min. Surface binding is expressed in terms of changes in response units (RU) with 1 RU being approximately 1 pg/mm². Sheep polyclonal anti-uromodulin antibody (300 nM) in PBS-Tween was immobilized by amine coupling to the chip surface activated with

Nephrology Dialysis Transplantation

aqueous solutions of 0.4 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 0.1 M N-hydroxysuccinimide for 300 sec at 5 μ L/min flow rate. For determination of kinetic constants, a dilution series of four concentrations (19 nM, 39 nM, 78 nM, 156 nM) of uromodulin was

loaded on 10 % acrylamide gel and analyzed as described above.

Immunohistochemistry

1
2
3 injected using the T100 in multichannel mode. The reference channel used in parallel did not
4
5 contain immobilized antibody, in order to detect background response and unspecific binding of
6
7 analyte to the surface. Between two measurements, the surface was regenerated by injecting
8
9 twice 10 mM glycine at pH 2 for 30 sec, which completely removed uromodulin from the
10
11 antibody. For data evaluation, the measured sensorgrams were referenced twice, first by
12
13 subtracting the signal from the reference channel, and second by subtracting the signal obtained
14
15 from injected pure buffer solution. Kinetic curves were evaluated using Biacore T100 Evaluation
16
17 Software (v. 2.0.2). A global fit was performed using the entire concentration series. Rate
18
19 constants for association and dissociation were calculated by taking a 1:1 binding model as a
20
21 basis.
22
23
24
25
26
27
28
29

30 Data analysis

31
32
33 Data were analyzed using Statistical Package for Social Sciences (SPSS) version 19 (IBM Corp.,
34
35 Armonk, USA). The Pearson correlation coefficient was used for correlation analysis, whereas
36
37 ANOVA and paired t-test were used for comparisons between the groups. A Bland-Altman plot
38
39 was used to evaluate agreement between uromodulin levels measured with the in-house ELISA
40
41 and commercial kit. Level of significance was set to $p < 0.05$.
42
43
44
45
46
47
48
49
50
51

RESULTS

Characterization of the antibodies against human uromodulin

The antibodies used for the in-house ELISA were characterized by immunoblotting and immunostaining (Fig.2). Immunoblot analysis of human urine and kidney samples in parallel with mouse kidney samples using the sheep polyclonal antibodies detected the uromodulin band at ~100 kD in all samples except the Umod KO kidney sample. The uromodulin band was also detected in human urine and kidney samples using the mouse monoclonal antibody (Fig.2A,top panel). Both the polyclonal and monoclonal antibodies also appropriately identified the deglycosylated and desialylated forms of uromodulin (Fig.2A,lowerpanels).

Staining of human and mouse kidney tissue samples with the mouse monoclonal or the sheep polyclonal antibodies detected uromodulin in the TAL, where it colocalized with NKCC2 at the apical surface area (Fig.2B). Surface plasmon resonance (25) was further used to characterize the uromodulin – antibody interaction (Fig.2C). As the isoelectric point of uromodulin ($pI = 3.2$) is too low for its immobilization to a carboxymethyl dextran surface, the capture sheep anti-uromodulin antibodies were immobilized at the surface of a sensor chip, and a dilution series of uromodulin was injected. For the interaction of immobilized sheep anti-uromodulin antibody with uromodulin, rate constants for association (k_{on}) and dissociation (k_{off}) of $4 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$ and $4 \times 10^{-4} \text{ sec}^{-1}$, respectively, were determined, giving a dissociation constant $K_D (= k_{off}/k_{on})$ of 10 nM. We also measured a strong binding response for the interaction of mouse anti-human uromodulin antibody to the sheep anti-uromodulin antibody - uromodulin complex. This situation

is comparable to the conditions in ELISA (see below). Regeneration of the surface with 10 mM glycine at pH 2.0 removed both the antibody and uromodulin.

Characteristics of the ELISA for uromodulin

When tested against purified human uromodulin, the in-house ELISA for human uromodulin showed a sensitivity (minimum amount of analyte which can be accurately detected) of 2.8 ng/mL and a linearity (correlation between concentration and optical density) of 1.0 ([Fig.3A](#)). The inter- and intra-assay variabilities were determined at 3.28% and 5.46%, respectively. The assay had a detection range between 3.9 and 500 ng/mL. When compared with other assays, the in-house ELISA showed a wider range of measurement and lower intra- and inter-assay variability than commercially available routine kits ([Table I](#)). There was a robust correlation ($r=0.905$, $p<0.001$) when comparing the in-house ELISA with the MD Bioproduct kit. The Bland-Altman plot showed that the mean difference between both methods was $-1.47 \mu\text{g/mL}$ (95% CI, -3.21 to $0.27 \mu\text{g/mL}$) ([Fig. 3B](#)).

Influence of processing of urine samples

Since uromodulin has a tendency to aggregate, we first investigated the potential influence of vortexing and centrifugation on the determination of uromodulin levels in human urine ([TableII](#)). Comparison of fresh samples assayed before and after vortexing revealed a more than 50% increase in uromodulin levels (unindexed uromodulin: 5.02 ± 0.66 vs. $11.03 \pm 1.67 \mu\text{g/mL}$, respectively, $p=0.001$; indexed uromodulin: 10.84 ± 0.54 vs. $15.90 \pm 1.45 \text{ mg/gr creat}$, respectively, $p=0.001$, $n=37$). Treating the urine samples with an usual centrifugation protocol (10 min, 3600 rpm) also showed a strong effect, since centrifugation yielded a significant decrease in unindexed (6.40 ± 0.63 vs. $13.27 \pm 1.18 \mu\text{g/mL}$, respectively, $p<0.001$) and indexed

(9.97 ± 1.43 vs. 15.66 ± 1.34 mg/gr creat, respectively, $p < 0.001$, $n = 53$) uromodulin levels.

Immunoblotting analyses ([Fig.4](#)) revealed that centrifugation was responsible for the precipitation of uromodulin in the pellet of cell debris. In comparison with the uromodulin band detected in fresh, non-centrifuged urine samples, the signal was strongly attenuated in the centrifuged urine sample while becoming apparent in the resulting pellet.

Alkalinization of fresh urine sample to pH 8.0 did not influence the determination of urinary uromodulin, as compared with untreated (mean pH 5.68 ± 0.19) samples (unindexed uromodulin: 19.25 ± 4.14 vs. 20.60 ± 5.24 $\mu\text{g/mL}$, respectively, $p = 0.179$; indexed uromodulin: 17.56 ± 2.28 vs. 18.34 ± 2.67 mg/gr creat, respectively, $p = 0.260$, $n = 14$). Likewise, acidification of urine samples to pH 2.0 did not result in a significant difference between values from untreated (mean pH 6.15 ± 0.59) samples (unindexed uromodulin: 10.01 ± 2.25 vs. 9.73 ± 2.07 $\mu\text{g/mL}$, respectively, $p = 0.621$; indexed uromodulin: 18.86 ± 8.26 vs. 19.10 ± 8.55 mg/gr creat, respectively, $p = 0.782$, $n = 8$).

Influence of storage conditions

In order to cast light on the influence of storage conditions on the stability of uromodulin, we compared values obtained in samples analyzed at baseline and after 1 week or 5 months storage at room temperature, $+4^\circ\text{C}$ and -20°C . As compared to baseline, storage at either room temperature or 4°C or even -20°C were associated with decreased levels of both unindexed and indexed uromodulin ([Table III](#)). Addition of protease inhibitors at time of collection had some

effect on the degradation of the samples conserved at -20°C, but not on those kept at +4°C. In any case, the addition of protease inhibitors was insufficient to prevent a significant decrease in the uromodulin levels as compared to baseline values. In contrast, 4-month storage at -80°C was not

respectively, $p=0.001$, $n=37$). Treating the urine samples with an usual centrifugation protocol (10 min, 3600 rpm) also showed a strong effect, since centrifugation yielded a significant decrease in unindexed (6.40 ± 0.63 vs. 13.27 ± 1.18 $\mu\text{g/mL}$, respectively, $p<0.001$) and indexed

associated with significant changes in uromodulin levels in untreated samples. Further analyses revealed a slight but significant decrease after 8 months storage at -80°C (baseline uromodulin: $23.73 \pm 1.57 \mu\text{g/mL}$ vs. 8-month: $20.13 \pm 1.17 \mu\text{g/mL}$, $p=0.023$, $n=142$). Freezing-thawing cycles (from -80°C to 0°C) showed no significant changes in the levels of urinary uromodulin as compared to baseline (TableIV).

DISCUSSION

Increasing evidence suggests that the level of uromodulin in urine could represent a useful biomarker for kidney function (3,26). In this study, we validated an efficient and cost-effective immunoassay and characterized the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in human urine. The urinary uromodulin levels were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage.

To develop our in-house ELISA we used commercially available anti-uromodulin antibodies and validated their specificity in human and mouse kidney and urine samples. Both antibodies evidenced the ~100 kD band corresponding to uromodulin on Western blot, either in native or deglycosylated/desialylated state. They also showed the typical distribution along with NKCC2 in the apical membrane of the TAL. We used plasmon surface resonance to determine the binding constant for interaction of the immobilized sheep anti-uromodulin antibody to uromodulin to 10 nM which is in the expected range for an antibody-protein interaction. The immunoassay standard curve showed linearity over a broad range of values, allowing the detection of uromodulin with high sensitivity and very low inter- and intra-assay variability. It must be noted that, in contrast with previous results based on immunoblotting (20), dilution of the samples with deionized water yielded similar results than with TEA buffer. All these features, combined with an excellent correlation with the most used commercial ELISA, substantiate the interest of our immunoassay with the advantage of low cost, wide range of detection, and low variability.

Our analyses revealed a striking effect of vortexing and centrifugation on the determination of uromodulin in the urine. These two procedures yielded variations reaching 50% of the levels obtained on control, unprocessed samples (TableII). These findings are clinically

relevant, because low levels of urinary uromodulin have been suggested to be of diagnostic value in UAKD (3, 9-11). The effect of vortexing confirms the importance of the aggregation of uromodulin molecules in the normal urine. Uromodulin is also known to cofractionate with exosomes (27), the recovery of which is increased by vortexing (28). Uto et al. previously suggested that uromodulin may be trapped in cell debris or aggregated with crystals (18) after centrifugation protocols that are usual to remove contamination due to lysis or suspended cells (29). Our data confirm these findings and show that centrifugation of urine may decrease the level of uromodulin by ~30%. Thawed urine samples should thus be vortexed but not centrifuged before assaying uromodulin.

The question of the stability of uromodulin during different storage protocols is critical for analyzing large, multicentric cohorts. Previous studies based on small sample size yielded inconsistent conclusions about the influence of storage duration and temperature (17-20). Furthermore, these studies did not take into account normalization for urinary creatinine, which is usual for kidney biomarkers - at least in a stable situation (29,30). Our results, obtained on a large number of samples, reveal that short (1 week) and longer (5 month) storage at room temperature, 4°C or -20°C causes a significant decrease in indexed urinary uromodulin levels, largely due to decreased uromodulin. In contrast, a 4-month storage at -80°C is associated with marginal, non-significant decrease in the unindexed and indexed values. Of note, the decrease in unindexed uromodulin levels becomes significant after a 8-month storage at -80°C. The fact that storage of untreated urine samples at room temperature, 4°C or -20°C significantly decrease the level of uromodulin substantiates the observations of Kobayashi et al (20). This effect is only partially attenuated with protease inhibitors, which show some effect for samples kept at -20°C - but insufficient to prevent a significant degradation. Taken together, these data confirm the fact that urine samples should be stored at -80°C and analyzed within 3 months to give the most reliable

1
2
3 measurements. Of note, up to 5 freezing-thawing cycles on ice did not affect the stability of
4
5 uromodulin stored at -80°C.
6
7

8
9 Previous studies also reported inconsistent results in terms of treatments (detergents or
10
11 TEA buffer, alkalization) supposed to solubilize aggregates of uromodulin in urine (17-20, 31).
12
13 Some of these treatments may interfere with the binding of uromodulin to the ELISA capture
14
15 antibody (18). We verified here that dilution with deionized water gave similar results than with
16
17 TEA, and that urine alkalization (or acidification) had no effect on the determination of
18
19 uromodulin. These data support the conclusion that dilution of the sample with water before the
20
21 assay, combined with vortexing, is an efficient way of disaggregation (31).
22
23
24

25
26 In summary, these data indicate that reliable uromodulin measurements can be obtained
27
28 from untreated urine samples, provided they are immediately stored at -80°C and assayed within
29
30 3 months, with vortexing and dilution with water to prevent aggregation. This methodology will
31
32 be useful for high-throughput analyses of uromodulin and its validation as a biomarker for renal
33
34 function and risk of CKD.
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52

53
54
55
56
57
58

52
53
54
55
56
57
58
59
60

attenuated with protease inhibitors, which show some effect for samples kept at -20°C - but insufficient to prevent a significant degradation. Taken together, these data confirm the fact that urine samples should be stored at -80°C and analyzed within 3 months to give the most reliable

ACKNOWLEDGEMENTS

These studies were supported in part by an Action de Recherche Concertée (ARC, Communauté Française de Belgique); the FNRS and FRSM; the Inter-University Attraction Pole (IUAP, Belgium Federal Government); the NCCR Kidney.CH program (Swiss National Science Foundation); the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 246539 (Marie Curie) and grant n° 305608 (EUREnOmics) and the Gebert Rief Stiftung (Project GRS-038/12). The expert assistance of N. Amraoui, H. Debaix, S. Druart, Z. Guo and S. Terryn is appreciated. We thank Dr. L. Rampoldi for providing the kidney extract obtained from the Umod KO mouse developed by Prof. X-R. Wu (Ref. 21), and Dr. S. Schauer for helpful discussions about the Biacore experiments.

The authors declare no competing financial interests.

REFERENCES

1. Koyner JL, Vaidya VS, Bennett MR, et al. Urinary Biomarkers in the Clinical Prognosis and Early Detection of Acute Kidney Injury. *Clin J Am Soc Nephrol* 2010; 5: 2154–2165
2. Tamm I, Hosfall FL. Characterization and separation of an inhibitor of viral haemagglutination present in urine. *Proc Soc Exp Biol Med* 1950; 74: 108-114
3. Rampoldi L, Scolari F, Amoroso A, Ghiggeri G, Devuyst O. The rediscovery of uromodulin (Tamm-Horsfall protein): from tubulointerstitial nephropathy to chronic kidney disease. *Kidney Int* 2011; 80: 338-347
4. Chabardès-Garonne D, Mejéan A, Aude JC, et al. A panoramic view of gene expression in the human kidney. *Proc Natl Acad Sci U S A* 2003; 100:13710-13715
5. Wiggins RC. Uromucoid (Tamm-Horsfall glycoprotein) forms different polymeric arrangements on a filter surface under different physicochemical conditions. *Clin Chim Acta* 1987; 162: 329-340
6. Serafini-Cessi F, Malagolini N, Cavallone D. Tamm-Horsfall glycoprotein: biology and clinical relevance. *Am J Kidney Dis* 2003; 42: 658-676
7. Renigunta A, Renigunta V, Saritas T, Decher N, Mutig K, Waldegger S. Tamm-Horsfall Glycoprotein interacts with Renal Outer Medullary Potassium Channel ROMK2 and regulates its function. *J Biol Chem* 2011; 286: 2224-2235
8. Mutig K, Kahl T, Saritas T, et al. Activation of the bumetanide-sensitive Na⁺,K⁺,2Cl⁻ cotransporter (NKCC2) is facilitated by Tamm-Horsfall protein in a chloride-sensitive manner. *J Biol Chem* 2011; 286: 30200-30210
9. Bollée G, Dahan K, Flamant M, et al. Phenotype and outcome in hereditary tubulointerstitial nephritis secondary to UMOD mutations. *Clin J Am Soc Nephrol* 2011; 6: 2429-2438
10. Dahan K, Devuyst O, Smaers M, et al. A cluster of mutations in the UMOD gene causes familial juvenile hyperuricemic nephropathy with abnormal expression of uromodulin. *J Am Soc Nephrol* 2003; 14: 2883-2893
11. Bleyer AJ, Hart TC, Shihabi Z, Robins V, Hoyer JR. Mutations in the uromodulin gene decrease urinary excretion of Tamm-Horsfall protein. *Kidney Int* 2004; 66: 974-977
12. Bernascone I, Janas S, Ikehata M, et al. A transgenic mouse model for uromodulin-associated kidney diseases shows specific tubulo-interstitial damage, urinary concentrating defect

and renal failure. Hum Mol Genet 2010; 19: 2998-3010

13. Padmanabhan S, Melander O, Johnson T, et al. Genome-wide association study of blood pressure extremes identifies variant near UMOD associated with hypertension. PLoS Genet 2010; 6: e1001177

14. Köttgen A, Glazer NL, Dehghan A, et al. Multiple loci associated with indices of renal function and chronic kidney disease. *Nat Genet* 2009; 41: 712-717
15. Chambers JC, Zhang W, Lord GM, et al. Genetic loci influencing kidney function and chronic kidney disease. *Nat Genet* 2010; 42: 373-375
16. Brunisholz M, Geniteau-Legendre M, Ronco PM, Moullier P, Pontillon F, Richet G, Verroust PJ. Characterization of monoclonal antibodies specific for human Tamm-Horsfall protein. *Kidney Int* 1986; 29: 971-976
17. Akesson I, Haugen H, Enger E. Quantification of uromucoid: a simplified method. *Scand J Clin Lab Invest* 1978; 38: 93-95
18. Uto I, Ishimatsu T, Hirayama H, Ueda H, Tsuruta J, Kambara T. Determination of urinary Tamm-Horsfall protein by ELISA using a maleimide method for enzyme-antibody conjugation. *J Immunol Methods* 1991; 138: 87-94
19. Torffvit O, Agardh CD, Kjellsson B, Wieslander J. Tubular secretion of Tamm-Horsfall protein in type I (insulin-dependent) diabetes mellitus using a simplified enzyme linked immunoassay. *Clin Chim Acta* 1992; 205: 31-41
20. Kobayashi K, Fukuoka S. Conditions for Solubilization of Tamm-Horsfall Protein/Uromodulin in Human Urine and Establishment of a Sensitive and Accurate Enzyme-Linked Immunosorbent Assay (ELISA) Method. *Arch Biochem Biophys* 2001; 388: 113-120
21. Thomas CE, Sexton W, Benson K, Sutphen R, Koomen J. Urine collection and processing for protein biomarker discovery and quantification. *Cancer Epidemiol Biomarkers Prev* 2010; 19:953-959
22. Reznichenko A, van Dijk MC, van der Heide JH, Bakker SJ, Seelen M, Navis G. Uromodulin in Renal Transplant Recipients: Elevated Urinary Levels and Bimodal Association with Graft Failure. *Am J Nephrol* 2011; 34: 445-451
23. Mo L, Zhu XH, Huang HY, Shapiro E, Hasty DL, Wu XR. Ablation of the Tamm-Horsfall protein gene increases susceptibility of mice to bladder colonization by type 1-fimbriated *Escherichia coli*. *Am. J Physiol Renal Physiol* 2004; 286: 795-802
24. Parsons CL, Rajasekaran M, Arsanjani AH, Chenoweth M, Stein P. Role of sialic acid in urinary cytoprotective activity of Tamm-Horsfall protein. *Urology* 2007; 69: 577-581
25. van der Merwe PA. Surface Plasmon Resonance in Protein-Ligand interactions: hydrodynamics and calorimetry, edited by S Harding and P Z Chowdhry. Practical Approach series, Oxford University Press 2001; pp.137-170
26. Köttgen A, Hwang SJ, Larson MG, et al. Uromodulin levels associate with a common UMOD variant and risk for incident CKD. *J Am Soc Nephrol* 2010; 21: 337-344

27. Hiemstra TF, Charles PD, Hester SS, Karet FE, Lilley KS. Uromodulin exclusion list improves urinary exosomal protein identification. *J Biomol Tech* 2011; 22: 136-145
28. Zhou H, Yuen PS, Pisitkun T, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. *Kidney Int* 2006; 69: 1471-1476
29. Waikar SS, Sabbiseti VS, Bonventre JV. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. *Kidney Int* 2010; 78: 486-494
30. Ortiz A, Sanchez-Nino MD, Sanz AB. The meaning of urinary creatinine concentration. *Kidney Int* 2011; 79: 791
31. Dawney AB, Thornley C, Cattell WR. An improved radioimmunoassay for urinary Tamm-Horsfall glycoprotein. *Biochem J* 1982; 206: 461-465

Table I. Comparison of the characteristics of the in-house ELISA for uromodulin and the available commercial ELISA kits.

Kit	Detection range (standard curve)	Inter-assay Variability	Intra-assay Variability
In-house	3.9 - 500 ng/mL	3.28%	5.46%
MD Bioproduct (Cat. M036020)	2.34 - 150 ng/mL	11.63%	8.36%
BioVendor (Cat. RD191163200R)	0.5 - 32 ng/mL	6.4%	2%
USCN Life Science Inc. (Cat. E96918 Hu)	3.13 - 200 ng/mL	<12%	<10%

Table II. Effect of sample processing (vortex, centrifugation) on the concentration of uromodulin in the urine.

	Unindexed Uromodulin ($\mu\text{g/mL}$)	P	Indexed Uromodulin (mg/gr creat)	P	N
Vortex	11.03 ± 1.67	0.001	15.90 ± 1.45	0.001	37
No vortex	5.02 ± 0.66		10.84 ± 0.54		
Centrifugation	6.40 ± 0.63	<0.001	9.97 ± 1.43	<0.001	53
No centrifugation	13.27 ± 1.18		15.66 ± 1.34		

Urine samples were vortexed for 10 sec. Centrifugation was performed for 10 min at 3600 rpm at room temperature. Two different sets of samples were used to test the influence of vortexing (N=37) and centrifugation (N=53).

Table III. Effect of storage conditions (duration, temperature, protease inhibitors) on the concentration of uromodulin in the urine.

		Unindexed Uromodulin ($\mu\text{g/mL}$)	P	Indexed Uromodulin (mg/gr creat)	P	N
1 week storage	Baseline	12.39 ± 2.41	0.078#	22.70 ± 3.35	0.014#	13
	RT	$6.14 \pm 1.34^*$		$13.00 \pm 2.34^*$		
	+4 °C	$7.22 \pm 1.60^*$		$11.49 \pm 1.50^*$		
	-20 °C	$9.98 \pm 1.96^*$		$18.69 \pm 3.18^*$		
4 month storage	Baseline	36.37 ± 2.62	0.354	24.05 ± 1.26	0.412	61
	-80°C	35.47 ± 2.32		23.30 ± 1.25		
5 month storage	Baseline	28.50 ± 6.76	0.068#	26.48 ± 3.45	0.001#	10
	+4°C	$10.17 \pm 3.96^*$		$10.27 \pm 2.35^*$		
	-20°C	$16.52 \pm 5.08^*$		$15.78 \pm 2.73^*$		
	+4°C & PI	$11.04 \pm 4.69^*$		$10.80 \pm 2.32^*$		
	-20°C & PI	$20.23 \pm 5.27^*\text{£}$	0.111#	$18.71 \pm 2.69^*\text{£}$	0.003#	

PI, treatment with protease inhibitors (Leupeptin and sodium azide). Three different sets of urine samples were used to assess influence of storage after 1 week (N=13), 4 months (N=61) and 5 months (N=10) vs. baseline levels. * $p < 0.05$ storage condition vs. baseline, £ $p < 0.05$ no vs. protease inhibitors, paired t tests; # ANOVA

Table IV. Effect of freezing-thawing cycles on the concentration of uromodulin in the urine.

		Unindexed Uromodulin ($\mu\text{g/mL}$)	P	Indexed Uromodulin (mg/gr creat)	P	N
Freezing- thawing cycles (-80°C to 0°C)	Baseline	22.96 ± 4.75	0.616#	18.14 ± 2.82	0.351#	8
	1	22.01 ± 3.81		20.19 ± 3.51		
	2	24.74 ± 4.05		23.67 ± 3.07		
	3	16.74 ± 2.68		18.17 ± 2.58		
	4	16.96 ± 3.36		16.20 ± 1.98		
	5	19.42 ± 4.56		15.91 ± 2.42		

ANOVA